Summary and Conclusions

Food-borne diseases represent a major public health and economic burden with an estimated ~2 million deaths in Asian countries. *Salmonella* Typhimurium is the leading causative agent of food-borne disease and is responsible for 49% of the outbreak. As this organism has impact on socio-economic level, hence its timely detection is essential.

Current detection methods for *Salmonella* include culture based, immunological and molecular methods, which are often cumbersome, time consuming and expensive. Most commonly used immunological assays are specific and involve the use of antibodies as recognition element. However, there are some limitations associated with antibodies, which restrain its usage. Polyclonal antibodies are not very specific while the monoclonal antibodies are costly and require skilled personnel for production. Instability towards environmental fluctuations like temperature and pH also limits the in-field applicability of antibodies. These limitations associated with antibodies warrants a search for substitute recognition element for detection of food-borne pathogens.

Phages are bacterial viruses, which are highly specific to their hosts (up to the level of serovar) and therefore, can be used as possible alternative recognition elements. Ease of propagation and longer shelf life are other important features of phages that stand out when compared to antibodies.

Another prime difficulty associated with current bacterial detection methods is the requirement of a well-equipped laboratory. This precludes detection of food-borne pathogens under field conditions and in rural locations. Development of on-site, field-usable detection device for detecting bacterial contamination is thus an unmet need.
Microfluidic technology has brought a new revolution in the field of on-site detection. The focus of microfluidics is to increase the sensitivity, specificity and reduce cost. This technique improves analytical performance by reducing the reagent consumption as well as analysis time. Furthermore, it also allows the miniaturization and helps to make the device portable.

Given the above background, in the present PhD work it was proposed to develop a portable microfluidics-based biosensor for detection of food-borne pathogen (viz *Salmonella* Typhimurium).

The specific objectives of the work were as follows:

**Objectives**

1. To develop an immuno bio-sensing method for the detection of bacteria (model organism *S. Typhimurium*) using bacteriophage (P22).

2. To design a prototype of microfluidic biosensor and its evaluation for detection of *S. Typhimurium* in spiked or actual food samples.

3. To demonstrate the use of phage display technique and other phage based techniques for bacterial detection.

The work carried out and results obtained are summarized below:

**Wild type phage (P22) as a recognition element**

For the detection of *S. Typhimurium*, a standard wild type bacteriophage P22 was used. Host specificity of P22 phage was investigated over a wide range of bacterial cultures using spot test analysis. P22 was found to be highly selective for *S. Typhimurium*, confirming its host specificity. Further, stability of P22 phage was also assessed and it showed a remarkable stability over a range of pH (5-10), and temperature (< 60°C for 60 min). Unlike many
phages, P22 infected its host in both log- and stationary- growth phase to produce a high titer of progeny phages ($10^{10}$ - $10^{12}$ PFU/mL of lysate).

After confirming the specificity and stability, phage based detection assay was optimized with respect to phage concentration, temperature and incubation time. Phage based assay was performed in ELISA format. For the assay, different dilutions of S. Typhimurium ($10^4$-$10^8$ CFU/100 μL) were immobilized in the wells of the ELISA plate. P22 phage and anti-phage antibody conjugated with HRP were used as primary and secondary recognition elements, respectively. For comparison, standard monoclonal antibody based assay was also performed. Observed increase in absorbance as a function of bacterial density confirmed that the phage-based assay was working. Importantly, at all the tested bacterial cell densities, the absorbance values with P22 phage were higher than the values obtained with monoclonal antibodies, suggesting higher sensitivity. The observed limit of detection for phage-based assay was $10^4$ CFU/mL. Specificity of the test was confirmed using E. coli, which showed a negligible absorbance signal in the phage-based assay.

**Translation of phage-based assay to a microfluidic platform**

To increase the sensitivity of the phage based assay and to make a portable detection device the method was translated to a microfluidic platform. For this purpose, a microfluidic assay chip was fabricated and components essential for translation of assay were optimized.

**(a) Fabrication of microfluidic assay chip**

A microfluidic channel of 500 μm diameter was fabricated using soft lithography method in poly (dimethyl siloxane) (PDMS) elastomer. Briefly, the method included (1) clamping of copper wires of 500 μm diameter (2) pouring of liquid PDMS (base: curing agent; 10:1) into the mold (3) curing of the polymer at 70°C for 120 min (4) removal of copper wire and de-molding to get
the microfluidic channel. Inlet and outlet of the capillary (diameter, 1.7 mm) were specially designed to avoid physical blockage of assay materials.

(b) **On-chip magnetic separation of bacteria**

In order to separate and immobilize the pathogens in microfluidic assay channel, magnetic nanoparticles based capture was employed. For magnetic separation, amine functionalized Fe₃O₄ nanoparticles (A-MNPs) were used. On-chip capture was optimized with respect to particles concentration and capture time. Locally purchased electromagnets were used for the separation purpose and optimized for the maximum field strength by altering the distance from the assay channel. It was found that at a distance of 0.5 mm, the field strength was 400 Gauss, which was sufficient to capture maximum number of in-flow particles. During on-chip experiments, capture efficiency obtained was typically ~99%.

(c) **Conjugation of anti-phage antibodies with quantum dots as detection labels**

Post magnetic capture, a fluorescent label was necessary for on-chip optical detection of pathogens. For this PDMS coated cadmium telluride (CdTe) quantum dots were conjugated with anti-phage an antibody, which was confirmed by agarose gel electrophoresis and FTIR studies.

**Microfluidic biosensor**

After optimizing different components and parameters of phage based assay suitable for a microfluidic platform, a biosensor was designed integrating magnetic separation of bacteria and subsequent in-flow detection.

a) **Designing of microfluidic biosensor**

Biosensor designed included three units, viz. electromagnetic, microfluidic and optical.
(i) Electromagnetic unit included three electromagnets (core diameter of 0.5 cm) separated by a distance of 1 cm. The electromagnets were connected in series and each represented a specific zone in which bacteria were captured with the aid of MNPs.

(ii) Microfluidic assay chip consisted of a single assay channel (500 μm diameter) placed in alignment with electromagnetic unit. Thus, under influence of magnetic field, channel could be divided into three zones, which were designated according to their functions viz., a) capture, b) recognition and c) detection. Inlet of the assay channel was connected with the tube (outer diameter, 1.8 mm) that was filled with reagents in a specific order, separated by air plugs. During assay, the assembly was connected with a syringe pump (Harvard instruments) to maintain a constant flow rate of 20 µL/min.

(iii) Optical detection unit integrated excitation light source (UV-LED, 350 nm), collimating assembly [a ball lens, condenser lens, band pass filter (542 ±10 nm) and avalanche photodiode to detect the signal. For detection, a fiber optic probe directed the excitation wavelength to the detection zone. Additionally at the bottom of the optical unit, a vibration device was placed to avoid agglomeration of MNPs during the assay.

b) Methodology for the detection of bacteria

The novel technique relied on sequential switching ‘on-and-off’ of electromagnets that lead to ‘capture, release and recapture’ mechanism of magnetic nanoparticles at various zones (capture, recognition and detection) under the influence of electromagnets. Briefly, the method included following steps; in the first step, Salmonella bound to MNPs were confined to capture zone upon activation of the electromagnet. Subsequently, with the introduction of recognition element i.e. phage, the magnet from capture zone was deactivated and that from recognition zone was activated. This allowed interaction of Salmonella-MNPs with phage and the resulting complex was captured in the second zone. Similarly, in the third step, magnet from...
recognition zone was deactivated and detection moiety was allowed to interact with *Salmonella*-MNPs-phage complex. Here magnet in the detection zone was activated which captured the complex and the signal was detected with the optical detection unit.

c) *Testing of microfluidic biosensor*

Phage-based assay was performed with $10^1$-$10^7$ CFU/mL of *S. Typhimurium*. The fluorescence intensity increased linearly with increase in cell concentration, thus confirming in-flow capture and detection of bacteria. The limit of detection of phage-based assay in microfluidic chip was tested with milk and was found to be $10^3$ CFU/mL. The assay was completed in 27 min. The increased sensitivity in this assay as compared to ELISA format phage-based assay could be attributed to increased accessibility of bacteria to the surface of MNPs during capture. Moreover, the methodology developed helped in faster detection of bacteria.

**Phage displayed peptides as an alternative recognition element**

The work proved that wild type phages were advantageous over antibodies as recognition element. However, the process of screening for an appropriate phage is tedious and time consuming; and naturally occurring bacteriophages may have a broad host range. Further, wild type phages are prone to mutation, which may limit its in-field applicability. To address these issues, phage displayed peptides as an alternative recognition element was explored. Phage display library is a random peptide library that allows the identification of peptide that binds with the target of interest with high specificity. In this study phage display random dodecapeptide library (PhD-12) (New England Biolab, USA) was used and subjected to biopanning against LPS of *S. Typhimurium*. According to the manufacturer’s protocol, after four rounds of biopanning, 30 phage clones that were obtained on IPTG-X-gal agar were selected randomly and amplified. Subsequently, DNA from each clone was isolated, sequenced and decoded to yield a peptide sequence (NEB cutter software).
Based on amino acid sequence and the frequency of occurrence, all the clones were classified into four classes. First class contained the peptides that differed in only one or two amino acids. All the sequences from the first class were screened by ELISA with LPS (according to standard procedures), and the sequence giving highest signal was selected and compared further with sequences from other classes following the same procedure. Two peptides that showed high affinity towards LPS in ELISA were further characterized by performing Surface Plasmon Resonance analysis. The dissociation constants were calculated to be $2.06 \times 10^{-8}$, and $2.38 \times 10^{-8}$ respectively that confirm their affinity towards LPS.

The selected peptides were tested against intact S. Typhimurium cells by ELISA. Phage displayed peptide (pep49: NFMESLPRLGMH) provided the highest signal with low cross reactivity. Using phage display peptide as recognition element, an assay was performed for the detection of S. Typhimurium over the range of $10^2$-$10^7$ CFU/mL. The observed limit of detection with phage-displayed peptide was found to be $10^3$ CFU/mL.

The interaction of selected peptide with LPS was determined using molecular dynamic (MD) simulations. Structure of pIII protein of M13 phage (the ligand) was extracted from Protein Data Bank (PDB ID: 1G3P), and sequence of selected peptide along with a spacer (GGGS) is appended at N-terminal. This model closely mimicked the experimental conditions. A homology model of the protein with the peptide was generated using the MODELER program (version 9.7, Discovery Studio). The structure showing lowest Discrete Optimized Protein Energy (DOPE) score was selected and MD simulation was performed for 50 ns. Frames for only the N-terminal 12 residues were written out as separate PDB files at intervals of 5 ns.

Similarly, crystal structure of tetrascarbohydrate unit consisting of rhamnose-mannose-(abequose)-galactose (representing the O-antigen of LPS, the receptor) was extracted from protein Data Bank (PDB ID: 1MFE) and MD simulations were performed.
Each frame representing unique conformation of the peptide was docked with the O-antigen of LPS using GLIDE. Docking score for all the conformation of the peptide with the LPS was in the range of -3.2 to -4.6 confirming high affinity towards its target. Further analysis suggested that leucine and arginine residues of selected peptide interacted with the abequose and mannose with the residue of the O-antigen of LPS, respectively and, therefore, showed specificity towards Salmonella species.
Salient results and findings:

- Utility of wild type P22 phage as specific and stable recognition element for detection of *Salmonella* Typhimurium was demonstrated.

- Novel PDMS coated CdTe quantum dots were synthesized and used as detection labels to develop a phage-based assay in ELISA format.

- A novel method for the fabrication of a circular microfluidic channel with different dimensions was successfully developed.

- In-flow capture of bacterial cell in microfluidic chip using amine functionalized magnetic nanoparticles was demonstrated.

- A prototype portable microfluidic biosensor was designed, which integrated simultaneous bacterial separation and detection using novel ‘capture and release’ mechanism.

- Designed prototype biosensor was successfully tested for the fluorescence based optical detection of *S. Typhimurium* from milk.

- Phage displayed peptides were successfully used as alternative recognition elements for the detection of *S. Typhimurium*. 
Conclusions

The results obtained in the present study enable us to conclude as follows:

1. It is possible to utilize wild type bacteriophage (P22) as a specific and stable recognition element for the detection of food-borne pathogens (S. Typhimurium).

2. Prototype microfluidic biosensor developed as part of the study, which integrates magnetic separation of bacteria (S. Typhimurium) in microfluidic channel and its in-flow live optical detection is a novel concept. The developed platform technology opens up many possibilities to use microchannels for bacterial detection. This technology is likely to lead to the development of inexpensive methods for screening and detection of a variety of clinical, water, food, and environmental samples for multiple organisms.

3. Genetically engineered phages (phage-displayed peptides) prove to be a robust and inexpensive alternative to antibodies, as recognition elements.

*To the best of our knowledge, this is the first study of its kind that demonstrates the utility of incorporating phages in a microfluidic platform for the detection of bacteria. The study also highlights the benefits of integrating key features of seemingly unrelated entities (e.g. bacteriophages and microchannels) in the development of novel and useful technologies.*

*In light of the above findings, the present work significantly contributes to the existing knowledge and paves the way for future research.*